

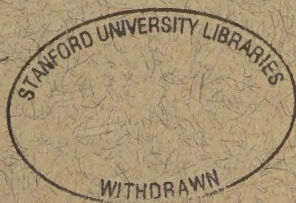
THE EFFECT OF SALTS ON THE PARACASEIN-
SODIUM HYDRONIDE EQUILIBRIUM

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PHYSICO-CHEMICAL STUDIES ON PROTEINS

VI. The Effect of Salts on the Casein-Sodium Hydroxide and Paracasein-Sodium Hydroxide Equilibria*

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The nature of the reaction of acids and bases with proteins has been studied by numerous investigators. Conflicting conclusions have been drawn from their work concerning the relative importance of colloidal and chemical forces in these reactions. Gortner¹ sums up the present attitude toward the problem in a statement that there are three schools of thought: One holds to the view that acid- and base-binding by proteins may be explained in terms of strictly chemical forces, another believes that surface energies are involved and a third group maintains that both colloidal and chemical forces play a part. Hoffman and Gortner² favor this view. Their work indicates a chemical type of combination between hydrogen ion concentrations of pH 2.5 and pH 10.5. Above or below the limits of this range their evidence indicates an adsorption type of combination.

Adequate reviews of the work in this field have been given by Robertson,³ Loeb,⁴ Hoffman and Gortner,² Cohn,⁵ and Gortner.¹ More recent contributions are those of Bancroft and Barnett.⁶

Our attention is called at the outset to the number of different methods which have been used to measure the extent of acid- or base-binding by proteins. It is obvious that no single method can of itself tell whether such binding is chemical or colloidal in nature. Such an inference can be made only after giving consideration to the amounts bound under the different sets of conditions. The relatively large number of methods employed over a short span of years is in itself evidence that the problem is far from a complete solution.

Because it was deemed impracticable to separate a protein "salt" from solution in sufficient purity for analysis, as was attempted by Van Slyke and Hart⁷ with "calcium caseinate" and Spiro and Pemsels⁸ with "sodium caseinate," due to alteration in the "salt" during coagulation, investigators have had recourse to indirect methods whereby a measurement of the amount of acid or alkali bound by a protein could be effected. The protein is treated with acid or base in the quantities and under the conditions determined by the experimenter; and the concentration of acid or alkali remaining "unbound" is determined by one of several methods. F. A. Hoffman⁹ measured the catalytic

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effect of the unbound ions on hydrolysis reactions, Sjöqvist¹⁰ determined the change in electrical conductivity, while Bugarszky and Liebermann¹¹ studied the freezing point depression and made potentiometric measurements of the unbound acid or alkali.

The potentiometric method has been deemed the most reliable and has been employed by most subsequent workers. In such a determination it has been generally assumed that the magnitude of the measurement is in no way influenced by the presence of the protein "salt." In other words, the same amount of unbound acid or base in the same volume of aqueous solution should give exactly the same potentiometric reading. This assumption is of doubtful accuracy. A really satisfactory measurement would be one that would not be interfered with by other substances in solution, or would be one to which corrections could be applied for the effect of the other substances which are present.

It has been definitely shown that the alkali bound by protein increases with rising pH and that the acid bound increases with decreasing pH. Gortner¹ points out that this dependence on pH of the amount of reaction occurring suggests that forces of adsorption are involved in the reaction. But Robertson³ states that this is no proof that proteins do not obey the law of constant combining proportions, and points out in defense of his claim that the same change occurs among complex and double salts and the salts of polybasic acids or polyacid bases in the domain of inorganic chemistry. We are asked to visualize a molecule with many acid groups, only part of which ionize in lower concentrations of alkali, but more of which ionize in higher concentrations. Accordingly we have a greater amount of alkali-binding in these higher concentrations because there are more acid groups in a reactive state. A similar explanation is offered for the effect of a lowered pH on the acid-binding of proteins. Loeb⁴ emphasizes that at the same pH a protein will bind equivalent amounts of the different acids or alkalis. One might expect that at an extremely high or low pH, where all the acid or basic groups are ionized, the amount of alkali or acid bound would approach a constant value. Such is not the case. If we are to accept the explanations of Robertson and Loeb, we must consider each protein molecule as having an indefinite number of potential acidic or basic groups differing almost insensibly from each other in their dissociation constants.

Attempts have been made to correlate the binding with the chemical composition of the protein. Greenberg and Schmidt,¹² using Hitchcock's¹³ data, have shown that the amount of acid bound by gelatin is essentially equal to the sum of the free amino nitrogen plus the amino group of arginine. Cohn and Berggren¹⁴ calculate that the casein molecule contains from nineteen to twenty-eight free acid groups, and that casein combines with no more base than is required to neutralize them. Cohn⁵ points out that the more diamino acids a protein contains the more acid it will bind, whereas the more dibasic acids it contains the more alkali it will bind. All this is suggestive of chemical combination, but it does not provide definite proof that adsorption does not play a rôle. Furthermore these investigators worked within a rela-

tively narrow range of pH. With alterations in the systems decided changes in the amounts of acid or base bound might be revealed.

Evidence in favor of an adsorption reaction is seen in the relation between the amounts of alkali or acid bound as related to the original concentration. When graphed such data yield what appears to be an adsorption curve. This has been shown by Tolman and Stearn¹⁵ using fibrin and hydrochloric acid, Herzog and Adler¹⁶ using hide powder, Procter¹⁷ using gelatin, Tolman and Bracewell¹⁸ using fibrin and alkalies, and Izaguirre¹⁹ using the data of Lloyd and Mays²⁰ on hydrochloric acid and gelatin. Weber²¹ did not find this to be true for fibrin and acids.

Van Slyke and Hart⁷ and Van Slyke and Van Slyke²² pointed out that casein binds more acid at lower temperatures than at higher ones. That is, the binding has a negative temperature coefficient. This is not typical of chemical reactions, but is typical of adsorption. The data of Fischer and Hooker²³ with casein reveal the same fact. Weber did not find the reaction between fibrin and hydrochloric acid to have a negative temperature coefficient. Hoffman and Gortner² found a marked negative temperature coefficient of the acid- or alkali-binding at the higher concentrations of acid and alkali than are represented by pH 2.5 and pH 10.5. Within this range the amount of binding was influenced by the chemical composition of the protein; outside the range all proteins showed the same binding capacity, irrespective of their chemical composition. Indeed, when the original concentration was 0.500 normal "there is more alkali bound . . . than can be accounted for by chemical combination assuming that there is an available carboxyl group for each nitrogen atom, an assumption far in excess of possibility." This would mean that within the range pH 2.5 — pH 10.5 the acid and alkali added are neutralized by the amino and carboxyl groups. At pH 2.5 or 10.5 all of these have reacted, and any further reaction must be due to adsorption on the protein micelles. Thomas and Mayer²⁴ found that if acid were added to gelatin the interferometer readings increased in a straight line ratio until a "stoichiometric" point was reached at which the rate of increase became larger but remained a straight line function. This might be interpreted as confirmatory evidence for the theory of Hoffman and Gortner.

Bancroft and Barnett⁶ have made phase rule studies of the proteins. Known amounts of gaseous ammonia or hydrogen chloride were added to dry proteins and the partial pressure of the gas at equilibrium was measured. The pressure-concentration curves for these gases with solid acids or bases showed clearly whether or not definite chemical compounds were formed. A stoichiometric reaction was shown by the appearance of a flat in the curve during which time the solid acid or base was reacting with the gas to form a chemical compound. When applied to proteins it appeared that casein, arachin, zein, fibrin and gliadin adsorb ammonia readily with no evidence of the formation of any chemical compound. Casein, arachin, fibrin, gliadin and edestin did form definite compounds with hydrogen chloride. Zein did not form a chemical compound with hydrogen chloride. The graphs for those

proteins which did form definite chemical compounds indicate that they subsequently adsorb more gas than was necessary for the formation of initial chemical compounds.

The Problem

A new method of attack in the problem of acid and alkali binding is suggested by the work of Kolthoff and Bosch²⁵ on the influence of neutral salts on acid-base equilibria. By determining potentiometrically the pH of successive dilutions of equimolar mixtures of a weak acid and one of its salts they were able to calculate the dissociation constant of the acid, using the Debye-Hückel equation. On the addition of neutral salts to one of the dilutions, changes in pH were noted which were shown to be due to an increase in the activity coefficient of the undissociated acid. The application of this method to protein study is based on the hypothesis that a protein is a weak acid. However the calculations of Kolthoff and Bosch involved the degree of basicity of the acid, its molecular weight and its average ionic size. None of these values are known with any degree of certainty for proteins. So the thermodynamic study as applied to weak acids presents difficulties when applied to proteins. It should be possible however to make similar investigations using a protein as a weak acid, and to judge from the observations recorded whether it behaves as a true electrolyte, or whether it reacts in a manner that can be explained only on the assumption that colloidal behavior is involved.

Both casein and paracasein were chosen for this investigation since we desired to ascertain whether a study of this kind would reveal any deep-seated difference in the chemical nature of the two proteins. Doubts that there is any such difference had already been expressed by Richardson and Palmer.²⁶

Experimental

Reagents.—Paracasein was prepared from skimmed milk according to the method of Van Slyke and Baker²⁷ and Van Slyke²⁸ for casein. Previous to the addition of acid the calcium was precipitated by the addition²⁹ of 0.5 cc. saturated potassium oxalate for every 100 cc. of milk and the precipitate removed by supercentrifuging. The milk was heated to 40°C. and rennet was added. The milk was kept at this temperature for a length of time determined by the time required for the formation of a firm curd when rennet in the same concentration was added to some of the milk from which the calcium had not been removed. Precipitation with acid and solution in alkali for purification then followed the procedure of Van Slyke and Baker.

The protein so prepared analyzed as follows: 5.88% moisture, 0.072% ash, 14.80% nitrogen, 0.863% phosphorus, and 0.621% sulfur. The composition on a moisture-free basis was: ash 0.076%, nitrogen 15.73%, phosphorus 0.91%, sulfur 0.66%. This agrees well with analyses of casein and paracasein reported by Bosworth,³⁰ Geake,³¹ Hammarsten,³² Lehman and Hempel,³³ and Van Slyke and Bosworth.³⁴

Other protein preparations used included one of paracasein used by Richardson and Palmer²⁶ with an ash content of 0.04%. A sample of casein used by

Hoffman and Gortner² was also available. It had an ash content of 0.34%, most of which was porcelain dust derived from grinding the material in a porcelain ball mill. A sample of isoelectric gelatin, having an ash content of 0.13%, was also used.

Pure alanine was used for comparative purposes. The neutral inorganic salts were of "analytical" grade and were ground to a powder, dried for five or six hours at 100°C. and kept in a desiccator. Their aqueous solutions were tested for neutrality with indicators. Only the potassium iodide was not as pure as desired, apparently containing traces of potassium carbonate. The sodium hydroxide solutions used were prepared carbonate-free. Freshly redistilled water collected so as to be free from carbon dioxide was always used.

Apparatus and Methods.—The amount of alkali bound by the protein was determined potentiometrically and is expressed as gram equivalents bound per gram of protein. Measurements were made with a L. and N. Type K potentiometer, using a high resistance galvanometer, readings being made to a tenth of a millivolt. Bailey electrodes and a normal calomel half-cell were used. Appropriate corrections were made for any determinations which were run at temperatures other than 25.0°. All potentiometric readings were calculated to pH values, taking into account appropriate temperature corrections. The values for K_w were likewise corrected for temperature variations, a value of 0.2828 at 25° being used for the value of the calomel cell. The set-up was tested by the use of sodium hydroxide-succinic acid buffers. A series of these is reproduced in Table II as indicative of what may be expected in a system where only chemical combination is taking place.

The protein was made up in 50 cc. volumes of the required degree of alkalinity and salt concentration. Where large enough concentrations of protein were being used, these were weighed into 100 cc. Erlenmeyer flasks, and 0.1 normal carbonate-free base, aliquots of the salt solution, and conductivity water were added to bring the final volume to 50 cc. When the concentration of protein desired was too small to permit of direct weighing without introducing large errors, a stock solution was made in sodium hydroxide of a suitable concentration and aliquots were taken to be diluted subsequently to the desired volume. Stock salt solutions of 1.0, 0.1, 0.01, and 0.001 normality were prepared. When extremely dilute solutions of protein were required, a volume of 300 cc. was used.

The protein dissolved rather readily in solutions at pH 9.0 or 12.0. Difficulty was experienced in securing solution of the protein at a pH near the neutral point. For this work a stock solution of protein in weak alkali was prepared and shaken in a mechanical shaker for twenty-four hours. The final pH of this solution was less than 7.0. In all experiments where the pH of the system exceeded 7.0, the E.M.F. readings were always made within ten hours after the protein was wetted with alkali. According to Cohn and Berggren¹⁴ no appreciable amount of caseose is split off from casein in that time.

Calculations of the amount of alkali bound were made according to the equations of Cohn and Berggren:¹⁴

$$\text{pH} + \text{pOH} = \text{Log } (1/K_w)$$

$$\frac{(\text{OH}^-)}{(\text{NaOH})} = \gamma$$

$$\text{pOH} = \log [1/(\text{NaOH})\gamma] = \text{pNaOH} + \text{p}\gamma$$

$$\text{or pNaOH} = \text{pOH} - \text{p}\gamma.$$

and since $\text{pNaOH} = \log 1/(\text{NaOH})$ the concentration of the uncombined NaOH is readily calculated. The pH was determined from potentiometric measurements. $\text{Log } 1/K_w$, the solubility product of water was obtained from Clark's³⁵ tables. Values for $\text{p}\gamma$ were taken from Lewis and Randall,³⁶ using the average values given for γ for Na^+ and OH^- , and obtaining certain values by interpolation from the plotted curves. A table of such values is reproduced by Gortner.¹ From pNaOH the concentration of free sodium hydroxide can be obtained. This subtracted from the original concentration gives the amount "bound" by the protein present.

Certain assumptions are necessary in employing this method of calculation, and we are employing the same assumptions which were made by Cohn and Berggren,¹⁴ in order that our data may be directly compared with theirs. The values for γ were determined from electromotive force measurements in which the activity of the sodium hydroxide was affected by sodium chloride present. In our investigations protein "salts" were present. We therefore assume* for purposes of calculation (1) that the protein and sodium hydroxide react in stoichiometric proportions, (2) that the so-formed protein salt is completely dissociated, (3) γNa^+ of $\text{Na}^+ + \text{proteinate}^- = \gamma \text{Na}^+$ of $\text{Na}^+ + \text{Cl}^-$, (4) therefore, $\gamma \text{proteinate}^- = \gamma \text{Cl}^-$, (5) there is no adsorption of Na^+ or of NaOH on the micelles, and no effect of the ionic micelles on the γ of Na^+ .

In previous investigations^{2,37} it was assumed that the magnitude of the measurement was in no way influenced by the presence of the protein "salt." We now "assume" that the magnitude of its effect is the same as that of the sodium chloride in the concentration in which it was present in the measurements of the value of γ . A comparison of this method of calculation with that of Palmer and Richardson³⁷ and Hoffman and Gortner² using their data shows that the two methods agree closely in the lower ranges of alkali concentration, but differ in the higher ranges. At first this difference shows itself in the present calculations giving a slightly lower binding capacity figure, but as the alkali concentration is increased still more the lower values are given by the method of calculation used by Hoffman and Gortner and Palmer and Richardson. We cannot draw hard and fast conclusions from these observations save that differences are noted, particularly at higher

* In order that there may be no misunderstanding it is emphasized that these assumptions are made "for purposes of calculation" and for comparison with Cohn's data. I personally believe that no one of these assumptions is strictly correct. R. A. Gortner.

concentrations of alkali. Opinions may well differ as to which method of calculation is the more nearly correct. Both present obvious inadequacies.

The Effect of pH on the Alkali-binding Capacity of Casein and Paracasein.—It was desired to determine how the amount of alkali bound per gram of paracasein varied with the pH of the equilibrium mixture. Accordingly solutions were prepared in which the concentrations of protein and sodium hydroxide were varied so as to give a series covering a fairly wide range of alkalinity. Measurements and calculations were made as described. The results are recorded in Table I. Data on casein are included for comparison.

Similar determinations were made of the binding of sodium hydroxide by succinic acid. In Table II these results are detailed. The object of these series of measurements was to afford an opportunity to compare the binding by the proteins with the binding by an acid of known constitution, it being known in the latter case that the binding was strictly stoichiometrical.

Two things of significance are noted immediately. In the first place, the amount of alkali bound by succinic acid is essentially a constant. That is, throughout a wide range of hydroxyl ion concentration, succinic acid reacts in the same proportion with sodium hydroxide. This is to be expected as

TABLE I

Comparison of the Alkali-binding Capacity of Casein and Paracasein at Various Degrees of Alkalinity

Sodium Hydroxide Normality	Protein gms. per liter	Casein*		Paracasein*	
		Equilibrium pH	Equivalents of Alkali bound per gm. Protein $\times 10^5$	Equilibrium pH	Equivalents of Alkali bound per gm. Protein $\times 10^5$
0.001	1.0			8.833	99.15
0.006	6.0			9.397	99.44
0.020	10.0	11.230	169.0	11.213	174.68
0.040	20.0	11.411	180.9	11.371	181.89
0.060	30.0	11.487	184.5	11.412	185.8
0.050	20.0	11.863	195.3	11.863	193.0
0.075	30.0	12.003	198.4	11.959	201.4
0.100	40.0	12.083	202.5	12.056	203.4
0.060	20.0	12.118	200.5	12.091	198.6
0.040	10.0	12.125	202.8	12.102	205.1
0.050	10.0	12.289	208.3	12.260	204.5
0.075	20.0	12.335	208.6	12.325	206.0
0.100	30.0	12.369	210.9	12.356	209.6
0.060	10.0	12.407	212.7	12.393	209.7
0.075	10.0	12.531	227.6	12.503	219.7
0.100	20.0	12.536	230.2	12.544	213.6
0.100	10.0	12.667	253.6	12.669	236.7

* Detns. by A. D. R. (1929).

it is conceded that the reaction is strictly stoichiometrical. The amount of alkali bound by the proteins, however, *increases with increasing alkalinity of solution*. This is characteristic of the behavior of proteins. As pointed out previously it has been explained both on the assumption that colloidal forces are entering into the reaction, and that there is an increase in the number of acid groups available for chemical reaction.

The second fact to be noted in our data is that, under similar conditions of concentration and pH, casein and paracasein are very similar in their base-binding capacity. This is contrary to the generally accepted view. In fact the essential difference between the two proteins has always been stated to be the difference in their base-binding capacity. Yet two different workers,

TABLE II

The Alkali-binding Capacity of Succinic Acid at Various Degrees of Alkalinity, Temperature 25°

(Series A and Series B determined by different individuals—R.A.G. and A.D.R.—a year apart and using different solutions and different potentiometric equipment)

	Sodium Hydroxide	Succinic Acid per Liter*	Equilibrium pH	Equivalents of Alkali bound per gram of Succinic Acid** × 10 ⁵
	Normality	Grams		
Series A	0.020	1.1804	7.195	169.43
	0.040	2.3608	8.189	169.42
	0.050	2.3608	11.816	170.25
	0.075	3.5412	12.034	164.88
	0.100	4.7216	12.066	173.12
	0.040	1.1804	12.290	170.41
	0.050	1.1804	12.295	173.59
	0.075	2.3608	12.347	172.65
	0.100	3.5412	12.394	172.54
	0.100	2.3608	12.537	172.14
	0.100	1.1804	12.670	172.65
Series B	0.040	2.3608	10.166	168.51
	0.050	2.3608	11.810	167.38
	0.075	3.5412	12.021	166.21
	0.100	4.7216	12.059	173.69
	0.040	1.1804	12.115	175.53
	0.050	1.1804	12.295	173.59
	0.075	2.3608	12.347	172.65
	0.100	3.5412	12.407	173.86
	0.100	2.3608	12.557	183.70

* These quantities of succinic acid were chosen so that the "alkali bound" would be in the same range as that "bound" in the casein-paracasein series.

** Theory 169.43×10^{-5}

(R. A. G. and A. D. R.), working a year apart, but working in a parallel manner, secure almost identical values for the two proteins. We shall have occasion to refer to this later.

The Effect of Neutral Salts on the Amount of Alkali bound by Casein and Paracasein.—The main object of this study was to test the effect of the addition of various quantities of neutral salts to the protein sodium hydroxide systems. Measurements were made at four varying ranges on the pH scale, the amounts of protein and base being selected so as to obtain this degree of acidity or alkalinity. The salts used with both proteins were potassium chloride, potassium bromide, and potassium iodide. Some data are reported for the casein—NaOH systems where sodium chloride and lithium chloride were used. Rather unsatisfactory results were obtained with the potassium iodide experiments, due probably to the small amount of potassium carbonate contained in the supposedly pure salt. Therefore where figures on potassium iodide are given, it should be recognized that they are recorded merely for purposes of comparison with the effects of the other salts.

The systems were prepared by diluting a sodium hydroxide-paracasein solution with the proper amount of water and stock solutions of neutral salts so as to bring the final solution to the proper concentration. In this manner values were obtained at about pH 12, pH 9, pH 6, and pH 5.

The data for the salt effect at the highest pH are given in Table III. Data on the effect of dilution of the protein-base system of the same relative concentration as used in the "salt effect" studies are included, it being deemed desirable to have this available for purposes of calculation. Table III includes as well dilution data for a casein-sodium hydroxide system of the same concentration and the effect of potassium chloride and sodium chloride on such a casein system.

Certain features appear outstanding in these data. The effect of the three salts on the paracasein-sodium hydroxide system at this range of pH is practically identical. It is true that potassium iodide seems to exert a slightly different magnitude of effect than the others, but this is due probably to impurities in the salt as pointed out above. There does not appear to be a significant lyotropic series for the anions. Whether any significance should be attached to the fact that when the data are plotted the curves resemble those of the adsorption type, we shall leave until later to decide.

It would appear then that either there are unjustifiable assumptions in the method of calculation which was employed, or else that in some manner the presence of a neutral salt increases the base-combining capacity of a protein. Different neutral salts with a common univalent cation and different monovalent anions exert essentially equal effects in increasing this capacity. At a pH of approximately 12 it becomes noticeable in salt solutions as dilute as 0.0025 normal. Salt solutions weaker than this exert no appreciable

TABLE III
Dilution and Salt Effect Data for Casein- and Paracasein-Sodium Hydroxide Systems at pH 11 to pH 12.5

Salt Normality	Concentration of Protein	Sodium Hydroxide	Paracasein		Casein		Equivalents** of Alkali bound per gm. Protein $\times 10^5$
			pH	Equivalents* of Alkali bound per gm. Protein $\times 10^5$	pH	Temperature $^{\circ}\text{C}$.	
None	20	0.100	—	—	—	25	202.2
"	15	0.075	12.407	227.5	12.429	25	200.9
"	12	0.060	12.330	218.3	12.351	25	193.0
"	10	0.050	12.282	204.5	12.300	25	191.7
"	8	0.040	12.163	209.0	12.184	25	186.0
"	4	0.020	11.875	209.2	11.920	25	179.5
"	2	0.010	11.553	232.1	11.619	25	167.4
"	1	0.005	11.345	179.0	11.391	25	149.1
"	0.3333	0.00167	10.901	168.1	10.944	25	126.6
0.5000 N KCl	10	0.05	12.176	256.5	12.184	—	—
0.2500 "	10	0.05	12.209	247.4	12.195	—	—
0.1000 "	10	0.05	12.203	240.9	12.189	—	—
0.0500 "	10	0.05	12.231	234.3	12.200	—	—
0.0250 "	10	0.05	12.201	231.2	12.227	—	—
0.0100 "	10	0.05	12.212	224.3	12.208	—	—
0.0050 "	10	0.05	12.214	221.5	12.195	—	—
0.0025 "	10	0.05	12.278	203.9	12.197	—	—
0.0010 "	10	0.05	12.277	204.2	12.224	—	—

* Detns. by A. D. R. (1929).

** Detns. by R. A. G. (1928).

TABLE III (continued)
Dilution and Salt Effect Data for Casein- and Paracasein-Sodium Hydroxide Systems at pH₁₁ to pH_{12.5}

Salt Normality	Concen- tration of Protein	Paracasein			Casein		
		Sodium Hydroxide	pH	Equivalents* of Alkali bound per gm. Protein $\times 10^5$	pH	Equivalents* of Alkali bound per gm. Protein $\times 10^5$	Equivalents** of Alkali bound per gm. Protein $\times 10^5$
0.5000 N KBr	10	0.05	12.126	264.8	—	—	—
0.2500 "	10	0.05	12.154	249.1	—	—	—
0.1000 "	10	0.05	12.162	244.5	—	—	—
0.0500 "	10	0.05	12.177	236.0	—	—	—
0.0250 "	10	0.05	12.219	219.5	—	—	—
0.0100 "	10	0.05	12.224	216.2	—	—	—
0.0050 "	10	0.05	12.235	208.9	—	—	—
0.0025 "	10	0.05	12.135	217.9	—	—	—
0.0010 "	10	0.05	12.244	204.5	—	—	—
0.5000 N KI	10	0.05	12.092	282.5	—	—	—
0.2500 "	10	0.05	12.133	261.0	—	—	—
0.1000 "	10	0.05	12.184	230.8	—	—	—
0.0500 "	10	0.05	12.200	220.7	—	—	—
0.0250 "	10	0.05	12.212	213.1	—	—	—
0.0100 "	10	0.05	12.215	210.9	—	—	—
0.0050 "	10	0.05	12.235	197.3	—	—	—
0.0025 "	10	0.05	12.241	205.3	—	—	—
0.0010 "	10	0.05	12.228	202.5	—	—	—
0.5000 N NaCl	10	0.05	—	—	—	—	222.2
0.2500 "	10	0.05	—	—	—	—	211.6
0.1000 "	10	0.05	—	—	—	—	200.8
0.0500 "	10	0.05	—	—	—	—	199.4
0.0250 "	10	0.05	—	—	—	—	197.3
0.0100 "	10	0.05	—	—	—	—	193.1

* Detns. by A. D. R. (1929).

** Detns. by R. A. G. (1928).

Dilution and Salt Effect Data for Casein- and Paracasein-Sodium Hydroxide Systems at pH 8 to 9.6*

TABLE IV

Salt Normality	Concen- tration of Protein gm. per liter	Sodium Hydroxide Normality	Paracasein *		Casein	
			pH	Equivalents of Alkali bound per gm. Protein $\times 10^5$	pH	Equivalents** of Alkali bound per gm. Protein $\times 10^5$
None	20.0	0.02	—	—	—	—
"	10.0	0.0100	—	—	9.588	99.78
"	7.5	0.0075	—	—	9.607	99.44
"	6.0	0.0060	9.370	99.57	9.578	—
"	5.0	0.0050	9.397	99.44	9.574	—
"	4.0	0.0040	9.349	99.40	9.582	—
"	2.0	0.0020	9.298	99.34	9.550	99.86
"	1.0	0.0010	9.014	99.32	9.460	—
"	0.5	0.0005	8.833	99.15	9.300	—
"	0.167	0.000167	8.487	99.21	8.982	97.74
			7.653	99.67	8.183	98.50
0.500 N KCl	10	0.010	—	—	—	—
0.500 "	5	0.005	8.723	99.85	9.206	99.65
0.250 "	10	0.010	—	—	—	99.37
0.250 "	5	0.005	8.766	99.83	9.223	99.67
0.100 "	10	—	—	—	—	99.41
0.100 "	5	0.005	8.839	99.80	9.256	99.65
0.0500 "	5	0.005	8.927	99.75	9.309	99.36
0.0250 "	5	0.005	9.016	99.70	9.396	99.52
0.0100 "	5	0.005	9.123	99.62	9.476	99.45
0.0050 "	5	0.005	9.176	99.56	9.532	99.35
0.00250 "	5	0.005	9.272	99.46	9.555	—
0.00100 "	5	0.005	9.250	99.44	9.571	—
0.00050 "	5	0.005	9.286	99.41	—	—
0.00025 "	5	0.005	9.275	99.41	—	—

* Detns. by A. D. R. (1929).

** Detns. by R. A. G. (1928).

TABLE IV (continued)
Dilution and Salt Effect Data for Casein- and Paracasein-Sodium Hydroxide Systems at pH 8 to 9.6

Salt Normality	Concentration of Protein	Sodium Hydroxide	Paracasein*		Casein		
			pH	Equivalents of Alkali bound per gm. Protein $\times 10^5$	pH	Equivalents* of Alkali bound per gm. Protein $\times 10^5$	Equivalents** of Alkali bound per gm. Protein $\times 10^5$
0.500 N KBr	5	0.005	8.653	99.88	—	—	99.39
0.250 " "	5	0.005	8.762	99.85	—	—	99.41
0.100 " "	5	0.005	8.820	99.82	—	—	99.40
0.050 " "	5	0.005	8.973	99.75	—	—	—
0.025 " "	5	0.005	9.049	99.70	—	—	—
0.010 " "	5	0.005	9.133	99.64	—	—	—
0.0050 " "	5	0.005	9.218	99.54	—	—	—
0.0025 " "	5	0.005	9.264	99.51	—	—	—
0.0010 " "	5	0.005	9.311	99.43	—	—	—
0.0005 " "	5	0.005	9.279	99.40	—	—	—
0.500 N KI	5	0.005	8.972	99.84	—	—	99.33
0.250 " "	5	0.005	8.800	99.83	—	—	99.45
0.100 " "	5	0.005	8.825	99.82	—	—	99.39
0.050 " "	5	0.005	8.912	99.73	—	—	—
0.025 " "	5	0.005	9.019	99.67	—	—	—
0.010 " "	5	0.005	9.162	99.62	—	—	—
0.005 " "	5	0.005	9.227	99.55	—	—	—
0.0025 " "	5	0.005	9.206	99.49	—	—	—
0.0010 " "	5	0.005	9.267	99.42	—	—	—
0.0005 " "	5	0.005	9.276	99.41	—	—	—

* Detns. by A. D. R. (1929).

** Detns. by R. A. G. (1928).

TABLE IV (continued)
Dilution and Salt Effect Data for Casein- and Paracasein-Sodium Hydroxide Systems at pH 8 to 9.6

Salt Normality	Concen- tration of Protein	Sodium Hydroxide	Paracasein *		Casein		
			pH	Equivalents of Alkali bound per gm. Protein $\times 10^5$	pH	Equivalents* of Alkali bound per gm. Protein $\times 10^5$	Equivalents** of Alkali bound per gm. Protein $\times 10^5$
0.500 N NaCl	10	0.010	—	—	—	—	—
0.500 "	5	0.005	—	—	9.224	25	99.69
0.250 "	10	0.010	—	—	9.090	25	99.54
0.250 "	5	0.005	—	—	9.260	25	99.68
0.100 "	10	0.010	—	—	9.159	25	99.50
0.100 "	5	0.005	—	—	9.324	25	99.66
0.050 "	5	0.005	—	—	9.211	25	99.48
0.025 "	5	0.005	—	—	9.045	29	99.54
0.010 "	5	0.005	—	—	9.144	29	99.44
0.010 "	5	0.005	—	—	9.236	29	99.35
0.500 N LiCl	10	0.010	—	—	—	—	—
0.500 "	5	0.005	—	—	9.195	25	99.73
0.250 "	10	0.010	—	—	9.163	25	99.51
0.250 "	5	0.005	—	—	9.234	25	99.71
0.100 "	10	0.010	—	—	9.171	25	99.51
0.100 "	5	0.005	—	—	9.317	25	99.67
0.100 "	5	0.005	—	—	9.207	25	99.49

* Detns. by A. D. R. (1929).

** Detns. by R. A. G. (1928).

effect on the base-binding capacity of paracasein. From our study we would designate 0.001 normality to be the "fading out" point of the salt effect.*

Table IV shows the salt effect on the paracasein-sodium hydroxide and casein-sodium hydroxide systems at approximately pH 9. It includes dilution data as well. Here we have results very similar to those observed at the higher pH. The addition of neutral salts increases the base-binding capacity of paracasein, and the order of the increase appears superficially to resemble the typical adsorption curve. As before, the three salts seem to exert equal effects. The fading out point of the salt effect however is at a normality of 0.00025 to 0.0005 as compared with 0.001 at the higher pH. Salt concentrations which appear to have no effect on paracasein at pH 12 will cause it to bind more alkali at pH 9.**

In general the casein results agree with those for the paracasein systems. The actual figures are not the same, though nearly so. The amount of alkali bound by the paracasein is in general slightly greater than that bound by casein. While the fading out point was not determined for casein-sodium hydroxide systems, it would appear to be similar to that for paracasein.

Table V gives the dilution and salt effect data for casein and paracasein systems at a pH of approximately 6. It will be noted that whereas in previous dilution studies, dilution *lowered* the pH of a solution, in this case it *raised* it. This is due of course to the fact that while we have alkali present, it is not present in sufficient quantity to overcome the acidity of the protein. We are merely making an acid solution less acid by dilution.

The salt effect is similar to that in the previous series. The fading out point is reached at a salt concentration of 0.0001 normality, lower than in any of the previous studies. That is, the lower the pH, or perhaps the more nearly "neutral" the solution is, the more effect do small quantities of neutral salts produce.

For purposes of comparison similar studies were made on an amino acid-sodium hydroxide system, alanine being the amino acid used. The dilution and salt effect data are reproduced in Table VI. Measurements were also made of the salt effect of a neutral salt containing a divalent cation, calcium chloride. It was impossible to use this salt in paracasein studies, due to the formation of a curd of "calcium paracaseinate." Like the proteins, alanine binds more alkali in the presence of a neutral salt. The calcium chloride

* In most of the data which are reported for casein, the salt solutions were not sufficiently dilute to prevent a shift in the pH of the casein-NaOH system. A distinct "salt effect" persists at a salt concentration of 0.01 N and this "salt effect" fades out only as we approach 0.0010 N salt concentration. There is, therefore a greater "salt effect" in these casein-NaOH systems than Kolthoff and Bosch²⁵ found in their acid-base equilibria studies. However, the data which we do present is adequate to demonstrate that the paracasein-NaOH system is essentially identical in its behavior in this respect to the casein-NaOH system.

** Admittedly the excess "binding" due to the presence of the salt appears to be very small numerically; thus the values are 99.85×10^{-6} equivalents in the presence of 0.5 N KCl and 98.54×10^{-6} equivalents in the salt-free system. These extremes, however, represent a range in equilibrium pH values of 8.723 and 9.349 respectively, and accordingly represent readily measurable differences. There is no question but that these systems show a pronounced "salt effect."

TABLE V
Dilution and Salt Effect Data for Casein and Paracasein-Sodium Hydroxide Systems at pH 6 to 7

Salt Normality	Concentration of Protein gm. per liter	Sodium Hydroxide Normality	Paracasein *			Casein **		
			pH	Equivalents of Alkali bound per gm. $\times 10^5$	Temperature $^{\circ}\text{C}.$	pH	Equivalents of Alkali bound per gm. $\times 10^5$	
None	10.0	0.0050	—	—	27	6.657	49.9993	
"	7.5	0.00375	6.601	49.9993	—	—	—	
"	6.0	0.00300	6.622	49.9991	—	—	—	
"	5.0	0.00250	6.630	49.9988	27	6.703	49.9984	
"	4.0	0.00200	6.658	49.9984	—	—	—	
"	2.0	0.00100	6.713	49.9965	—	—	—	
"	1.0	0.00050	6.768	49.9925	—	—	—	
"	0.5	0.00025	6.743	49.9858	27	6.836	49.9793	
"	0.167	0.0000833	6.825	49.8492	27	7.235	49.8415	
0.50000 N KCl	5	0.0025	5.865	49.99981	27	6.080	49.99957	
0.25000 "	5	0.0025	5.930	49.99978	27	6.131	49.99948	
0.10000 "	5	0.0025	6.064	49.99970	27	6.238	49.99935	
0.05000 "	5	0.0025	6.166	49.99961	—	—	—	
0.02500 "	5	0.0025	6.273	49.99951	—	—	—	
0.01000 "	5	0.0025	6.399	49.99931	—	—	—	
0.00500 "	5	0.0025	6.479	49.99917	—	—	—	
0.00250 "	5	0.0025	6.540	49.99905	—	—	—	
0.00100 "	5	0.0025	6.581	49.99896	—	—	—	
0.00050 "	5	0.0025	6.562	49.99887	—	—	—	
0.00025 "	5	0.0025	6.571	49.99885	—	—	—	
0.00010 "	5	0.0025	6.587	49.99881	—	—	—	

* Data of A. D. R. (1929).

** Data of R. A. G. (1928).

TABLE V (continued)

Dilution and Salt Effect Data for Casein and Paracasein-Sodium Hydroxide Systems at pH 6 to 7

Salt Normality	Concentration of Protein	Sodium Hydroxide	Paracasein *		Temperature	Casein **	
			pH	Equivalents of Alkali bound per gm. $\times 10^5$		pH	Equivalents of Alkali bound per gm. $\times 10^5$
0.5000 N KBr	5	0.0025	5.843	49.99979	—	—	—
0.25000 "	5	0.0025	5.912	49.99977	—	—	—
0.10000 "	5	0.0025	6.043	49.99969	—	—	—
0.05000 "	5	0.0025	6.145	49.99960	—	—	—
0.02500 "	5	0.0025	6.242	49.99950	—	—	—
0.01000 "	5	0.0025	6.351	49.99936	—	—	—
0.00500 "	5	0.0025	6.438	49.99922	—	—	—
0.00250 "	5	0.0025	6.497	49.99911	—	—	—
0.00100 "	5	0.0025	6.551	49.99903	—	—	—
0.00050 "	5	0.0025	6.571	49.99889	—	—	—
0.00025 "	5	0.0025	6.600	49.99887	—	—	—
0.00010 "	5	0.0025	6.600	49.99882	—	—	—
0.10000 N KI	5	0.0025	6.038	49.99969	—	—	—
0.05000 "	5	0.0025	6.146	49.99960	—	—	—
0.02500 "	5	0.0025	6.234	49.99951	—	—	—
0.01000 "	5	0.0025	6.369	49.99936	—	—	—
0.00500 "	5	0.0025	6.442	49.99924	—	—	—
0.00250 "	5	0.0025	6.513	49.99914	—	—	—
0.500 N NaCl	5	0.0025	—	—	27	6.006	49.9996
0.250 "	5	0.0025	—	—	27	6.086	49.9995
0.100 "	5	0.0025	—	—	27	6.220	49.9994
0.500 N LiCl	5	0.0025	—	—	27	6.074	49.9995
0.250 "	5	0.0025	—	—	27	6.130	49.9995
0.100 "	5	0.0025	—	—	27	6.208	49.9994

* Data of A. D. R. (1929).

** Data of R. A. G. (1928).

effect exceeded that of potassium chloride. It is probable that the salt of a divalent cation has a greater effect on the alkali-binding capacity of a protein than has the salt of a monovalent cation, both salts having a common anion. It will further be noted that the theoretical equivalents of NaOH were bound only when essentially equivalent amounts of the amino acid and the alkali were present.

TABLE VI

Dilution and Salt Effect Data for Alanine-Sodium Hydroxide System at pH 9.2 to 10

Salt Normality	Alanine Normality	Sodium Hydroxide Normality	pH	Equivalents of Alkali bound per Mole of Alanine* $\times 10^3$
None	0.0200	0.0100	9.775	495.5
"	0.0150	0.0075	9.791	494.2
"	0.0120	0.0060	9.785	491.2
"	0.0100	0.0050	9.767	491.5
"	0.0080	0.0040	9.767	489.5
"	0.0040	0.0020	9.788	480.5
"	0.0020	0.0010	9.747	466.3
"	0.0010	0.0005	9.659	445.3
"	0.0003	0.00016	9.203	439.1
0.5000 N KCl	0.01	0.005	9.670	493.2
0.2500 " "	0.01	0.005	9.677	493.4
0.1000 " "	0.01	0.005	9.699	493.0
0.0500 " "	0.01	0.005	9.703	492.7
0.0250 " "	0.01	0.005	9.721	492.7
0.0100 " "	0.01	0.005	9.750	492.1
0.0050 " "	0.01	0.005	9.767	492.2
0.0025 " "	0.01	0.005	9.763	491.9
0.0010 " "	0.01	0.005	9.763	491.9
0.5000 N CaCl ₂	0.01	0.005	9.376	496.4
0.2500 " "	0.01	0.005	9.496	495.5
0.1000 " "	0.01	0.005	9.571	494.6

* Theory = 449.08×10^{-3} .

Studies were made of the effect of salts on protein-water systems. Any results obtained in these could not be reported in terms of the effect on the alkali-binding capacity of the protein. But increase in base-binding capacity is accompanied by a lowering of the pH of the system. Casein-water, paracasein-water, gelatin-water, and alanine-water systems were treated with varying amounts of potassium chloride and the effect upon the pH of the system determined. These results are recorded in Tables VII and VIII.

TABLE VII

The Effect of KCl on the pH of Protein-in-H₂O Systems

Concentration of Protein in Gm. per liter	KCl	Casein	Paracasein	Gelatin
	Normality	pH	pH	pH
2	0.5000	4.910	4.884	5.070
2	0.2500	4.931	4.917	5.014
2	0.1000	4.986	4.960	5.018
2	0.0500	5.003	4.976	5.088
2	0.0250	5.040	4.984	5.074
2	0.0100	4.959	5.035	4.923
2	0.0050	4.978	5.115	4.926
2	0.0025	4.999	5.180	4.962
2	0.0010		5.217	4.910
2	0.0005			4.955
2	0.0000	5.137	5.356	5.003
40	0.0000	4.738	5.104	
40	0.0100	4.832	4.978	

TABLE VIII

Effect of KCl on the pH of an Alanine-in-H₂O Solution

Conc. Alanine = 0.2 moles per liter

KCl	pH	KCl	pH
Normality		Normality	
0.500	6.635	0.025	6.730
0.250	6.668	0.010	6.710
0.100	6.697	0.000	6.800
0.050	6.721		

The addition of increasing amounts of potassium chloride lowers the pH of the alanine in water solution. This effect is similar to the salt effect on the protein-base system. A similar effect is indicated for the casein-water and paracasein-water systems, though all data are not in harmony with this generalization. Values which do not fall in line with the general trend are due probably to experimental errors of measurement. On repeating these studies a similar series of data was obtained, but with values of slightly different magnitude. It will be observed that the casein and paracasein impart different pH values of the water systems, that for paracasein being the higher. The magnitude of the salt effect seems to be of the same order for both proteins.

It seems impossible to draw any conclusions from the gelatin studies. There is no apparent relationship between salt concentration and direction or magnitude of change in pH.

Discussion

Base-binding Capacity.—The difference in base-binding capacity of casein and paracasein and of succinic acid has already been discussed at some length. The amount of alkali bound by casein and paracasein was found to increase with the equilibrium pH, whereas the amount bound by succinic acid was constant over a wide pH range. This would indicate that other factors than a stoichiometrical combination were acting—possibly an adsorption type of reaction between protein and alkali being added to a stoichiometrical binding. If this were due to increased ionization of the protein as an acid at higher pH values we might reasonably expect to find a point at which the protein ceases to bind larger quantities of alkali. That is, all the carboxyl groups would have reacted, and there would be no further salt formation between the protein and the alkali. From this point on, the amount of alkali bound per gram of protein would be the same, irrespective of the pH of the solution. This constant value was not reached, even though the systems were studied at pH 12.7. It would appear doubtful then if those who believe the reaction to be strictly stoichiometrical are justified in their stand. No amount of hypothetical argument will alter the fact that the curve for the alkali-binding of a protein over a changing pH range does not resemble the one for the alkali-binding of an organic acid of known constitution and which dissolves to form a true solution.

The Dilution Series.—The dilution data for the various systems are of interest. It is impossible to make use of them to calculate dissociation constants, as Kolthoff did, for reasons which have been previously stated. His first step was to determine the pH at infinite dilution, using the Debye-Hückel equation:

$$-\log f_1 = 0.5\sqrt{\mu}$$

where μ is the concentration. The value 0.5 may be employed if we are dealing with a monobasic acid, as acetic acid. For a dibasic acid, 1.5 would be used; for a tribasic one, 2.5; etc. Assuming paracasein does dissociate as an acid, we may let its degree of basicity be equal to $x + 1$. Then the equation would become

$$-\log f_1 = (x + 0.5)\sqrt{\mu}$$

Assuming that the dilutions were carried far enough, then the values for the greater dilutions, substituted in the above equation should yield values from which we might express the pH at infinite dilution in terms of the basicity of paracasein, or

$$\text{pH}^\infty = \text{pH} + (x + 0.5)\sqrt{\mu}$$

Thus from the dilution data for paracasein in Table III,

$$\text{pH}^\infty = 11.553 + (x + 0.5)\sqrt{0.01}$$

$$\text{pH}^\infty = 11.345 + (x + 0.5)\sqrt{0.005}$$

using these as simultaneous equations and solving for x , we find that $x = -7.6$, or the degree of basicity $= -6.6$. Similar calculations from other series of the dilution data yield values for $x + 1$ as follows:

	Paracasein	Casein
Table III	-6.6	-7.4
Table IV	-34.1	-31.3
Table V	-3.7	

In each case, the degree of basicity of the acid (paracasein or casein), has a negative value. This is a necessary consequence since the two terms of the expression for pH^∞ are smaller in one case than in the other. That is, a decreasing pH is accompanied by a decreasing value under the square root sign. These expressions then can be equal only if x is negative. Whereas we might expect more satisfactory results for alanine, we find it responding to our calculations after the manner of the proteins to give a degree of basicity of -8.3 . Alanine is known to be monobasic. It would accordingly appear that this method of calculation cannot be applied to amphoteric electrolytes.

From the dilution data we are led to draw two conclusions. First, our studies do not lend themselves to ready theoretical mathematical interpretation. This may be due to the fact that there is not the constant proportion between the reacting substances that we expect in a strictly stoichiometrical reaction, or it may be due to effects other than stoichiometrical combination. In fact, this lack of definiteness suggests strongly that classical chemical effects are not responsible for the whole of the reaction. It has been pointed out by Randall and his coworkers^{38,39} that thermodynamic method cannot be applied in heterogeneous systems. These observations throw doubt on the question of applying thermodynamic equilibrium calculations to our data.

Secondly, we have seen that the proteins "bind" different amounts of alkali, the amount which is bound depending both upon the concentration of the protein and the concentration of the alkali. This confirms other findings.

The Effect of Salts on the Reaction between Proteins and Sodium Hydroxide.

—The presence of salts, such as KCl, KBr and KI, increase the ability of the protein to "bind" sodium hydroxide and when the equivalents of alkali bound per gram of protein are plotted against the normality of the salt solution, the resulting curves approximate parabolas and indicate that adsorption may be involved in the increase. In order to test this possibility, the logarithms of salt normality were plotted against the logarithms of alkali bound per gram of protein. A representative curve is shown in Fig. 1. In this figure (paracasein-NaOH-KCl at pH 12), it is seen that the curve approximates a straight line, except for extremely small values of salt concentration. Accordingly it would appear that at this pH the increase in the alkali binding is probably due to adsorption.

Paracasein-NaOH-salts at pH 9 and paracasein-NaOH-salts at pH 6 did not yield straight lines, but rather sigmoid curves with a greater curvature

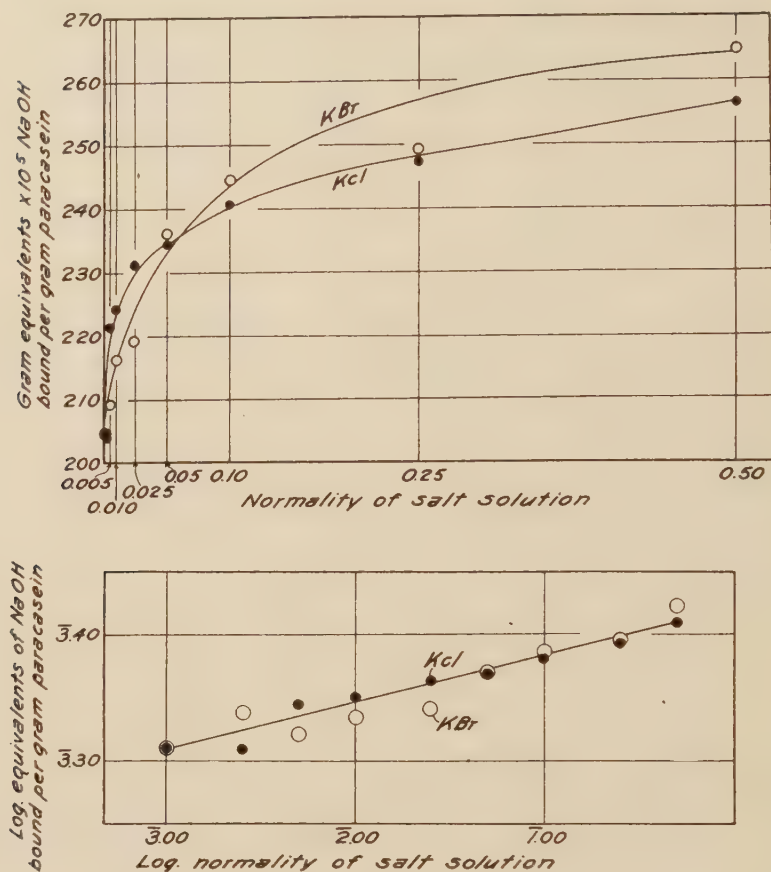


FIG 1

Showing the effect of various concentrations of KCl and KBr on the alkali binding of paracasein at approximately pH 12.

at pH 6 than at pH 9. It would thus appear that there is less of an adsorption effect at pH 6 than at pH 9 and less at pH 9 than at pH 12. We believe that there is certainly some adsorption at pH 9, and possibly some at pH 6.

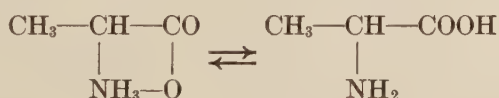
These findings appear to substantiate the findings of Hoffman and Gortner.² Adsorption is primarily concerned in the extra binding at pH 12, while more nearly classical chemical forces were involved at pH 9 and pH 6, more so in the latter than in the former. If the effect of added salts is to increase the activity of the undissociated molecule, as Kolthoff and Bosch found for certain acids, then the above is simple of explanation. The increased activity may result in increased power of adsorption and increased opening up of active groups. In the lower pH ranges where complete stoichiometrical binding has not occurred, there is further increase in the active groups followed by chemical reactions. Above pH 10.5, where the stoichiometrical binding is completed, the increased activity results in adsorption although some adsorption may occur below pH 10.5.

At lower pH values a shift in the equilibrium pH is observed with a lower salt concentration than at a higher pH. Kolthoff and Bosch²⁵ found that the salt effect in relatively simple acid-base systems disappeared at much higher concentrations of salt than those which we have found to show a decided effect in the casein- and paracasein-NaOH systems. Kolthoff,* after reviewing our data, comments on this fact as follows: "The striking effect of small amounts of neutral salts on the pH of the caseinate solutions shows definitely that they do not behave like simple acid-base systems of the classical type."

It should furthermore be emphasized that there is no definite lyotropic series for the anions Cl, Br and I in the "salt effect." Whether there is one for cations we did not determine. It is probable that a divalent cation would produce a greater effect than does a monovalent one.

This "salt effect" in a protein-NaOH system offers a logical explanation for the divergence which Hoffman and Gortner⁴⁰ found in a comparison of the "titration curve" and the "back titration curve" of a protein-NaOH-HCl system. They observed that when proteins were electrometrically titrated with sodium hydroxide (or calcium hydroxide) and then the mixture was back-titrated with equivalent amounts of hydrochloric acid, the "back-titration curve" invariably was different from the original titration curve, being shifted toward lower pH values. Due to the presence of the "salt effect" which we have noted, it is a necessary consequence that the second curve did not coincide with the initial one. Accordingly, the interpretation of pH measurements made in protein systems containing even small quantities of neutral inorganic salts is complicated by the concomitant "salt effect."

No explanation is offered for the increased reactivity of alanine with sodium hydroxide in the presence of salts. It can hardly be explained by an increase in the dissociation of alanine or by a shift of the internal salt equilibrium to the right:



Either the assumptions upon which our calculations are based are at fault, or else the amino group is in some unknown manner entering into the reaction. It cannot be through the formation of carbamino salts, since every precaution was taken to avoid the presence of carbon dioxide.

In the tables, data have been reported which were obtained from single preparations of casein and paracasein. That other preparations yield essentially identical data was proven by working with two other preparations of casein which have been used for physico-chemical studies in our laboratories. One of these samples was that used by Richardson and Palmer.²⁶ We also tested their sample of paracasein and secured values which were within experimental error of those which we report.

Therefore, as our contribution to the casein-paracasein problem, we must conclude that technic such as we have used shows little if any difference

* Private communication.

in these two proteins. Both bind approximately the same amounts of sodium hydroxide and both show essentially the same salt effect. If however we compare the dilution data for the two proteins as presented in Table III, we find that somewhat more alkali is bound by paracasein than by casein at similar concentrations of protein and alkali, or at the same pH. This is in harmony with the generally accepted notion of the reaction of the two proteins. Yet it is contrary to the findings presented in Table I. In general the systems reported in Table I were much more concentrated than were those reported in Table III. As a matter of fact only two of the systems had identical concentrations in both series, and the concentration of the system unquestionably affects the amount of base which is "bound" even though the ratio of protein:NaOH may be identical.

Summary and Conclusions

1. The amount of sodium hydroxide "bound" by casein and paracasein increased with the equilibrium pH. This suggests that adsorption may play a part in the reaction.
2. In similar studies succinic acid "bound" a constant amount of sodium hydroxide over an increasing pH range.
3. At pH 12.2 adsorption was probably the factor causing increased combination. At pH 9.0 and pH 6.0, binding by acidic groups appears to largely account for the results obtained.
4. The amount of sodium hydroxide "bound" by casein and paracasein is increased by the presence of potassium chloride, potassium bromide, or potassium iodide.
5. The addition of neutral salts causes a shift in the equilibrium pH of a casein-NaOH or a paracasein-NaOH system toward a lower pH value. This "salt effect" is much more pronounced in these protein-base systems than has been reported to be the case for simpler acid-base systems. Concentrations of neutral salts, as low as 0.001 N in some instances, produced a measurable pH shift.
6. The magnitude of the salt effect is essentially the same for potassium chloride, potassium bromide and potassium iodide. This "salt effect" exhibits no marked lyotropic series for these anions. These protein-NaOH systems are more sensitive to the addition of salts at a pH closer to their isoelectric points than at one further away.
7. This "salt effect" must be taken into consideration in the interpretation of the electrometric titration curves of proteins. If one titrates proteins electrometrically with either acid or alkali and then back-titrates the resulting system, the back-titration curve, due to the salt effect, will not coincide with the original titration curve. If a protein solution contains even small quantities of neutral inorganic salts, the equilibrium pH which is attained on the addition of a definite quantity of acid or alkali will be different from that which would be attained in the absence of the neutral inorganic salts.

8. In the pH ranges studied, and with the methods employed, casein and paracasein react essentially alike. The paracasein, in some instances, appears to bind slightly more alkali than does casein. Both respond in the same manner to the addition of salts.

9. This study affords evidence that a strictly classical thermodynamical treatment of the data of protein systems does not indicate a "maximum binding capacity" of NaOH by either casein or paracasein, such as is a necessary consequence of a stoichiometrical salt-formation, uncomplicated by adsorption reactions. The amount of sodium hydroxide which is bound by either casein or paracasein increases with increasing equilibrium hydrogen ion concentration in spite of attempts to apply assumptions of a strictly stoichiometrical combination to the calculation of the data.

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